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RECRUITMENT OF CIRCULATING CELLS INTO SKELETAL MUSCLE – OCCURRENCE AND POSSIBLE MECHANISMS

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RECRUITMENT OF CIRCULATING CELLS INTO SKELETAL MUSCLE – OCCURRENCE AND POSSIBLE MECHANISMS

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Tissue remodeling is a central process for all life. When a tissue is exposed to environmental stress such as injury, both local cells and cells from the periphery are recruited to regenerate and remodel the tissue. During the last decades the importance of resident cells, e.g. satellite cells (SaCs), and circulating cells, e.g. endothelial progenitor cells (EPC) and monocytes, in the process of skeletal muscle remodeling has been recognized. The aims of the current thesis were to investigate 1) whether cells from the bone marrow (BM) incorporate into skeletal muscle tissue in adult humans, 2) the impact of exercise on the level of circulating cells suggested to be important for skeletal muscle remodeling, and 3) if exercise induces the expression of cell recruiting factors in the skeletal muscle tissue. Four studies are included in the thesis. Women transplanted with male hematopoietic stem cells participated in study I. Study II and IV included healthy men and women that performed 60 min of cycling exercise, and study IV included controls. Study III was a training study in which 10 men performed one-legged exercise four times per week for 5 weeks with and without blood flow restriction. In all studies, muscle biopsies were obtained, and blood samples were collected in study II. Methods used in the studies included FISH, immunohistochemistry, flow cytometry, ELISA, qPCR, laser dissection and microdialysis. Y-chromosomes were found in muscle fiber and endothelial cell (EC) nuclei in skeletal muscle of women transplanted with male bone marrow. No Y-chromosomes were detected in the SaC niche. Directly after exercise, all monocyte subsets were significantly increased with the non-classical monocytes displaying the highest fold increase. At 2 h after exercise, only the classical subset was increased. The level of EPCs tended to increase with exercise. There was a significant increase in catecholamines, known to mobilize these cells with exercise, while no change was seen in the plasma levels of VEGF-A, G-CSF and SDF-1. The levels of endothelial adhesion molecules, E-selectin, ICAM-1 and VCAM-1, were all increased in skeletal muscle with exercise. VEGF-A expression was induced in skeletal muscle with the current exercise regime, and VEGF-A stimulation increased the level of endothelial adhesion molecules in HUVECs. An increase was observed for MMP-9 with one bout of exercise and MMP-2, MMP-14 and TIMP-1 with training, indicating different regulating mechanisms. The expression pattern and laser dissection did not support a myogenic origin of MMP-9, while MMP-2 mRNA was expressed in muscle fibers. The monocyte recruiting chemoattractant CX₃CL1 was increased after a single bout of exercise and was localized to the skeletal muscle endothelium. Moreover, microdialysate obtained from exercised muscle increased the expression of CX₃CL1 in HUVECs. THP-1 monocytes, primary human myoblasts and myotubes stimulated with CX₃CL1 increased their expression of pro-angiogenic and pro-inflammatory factors, supporting a role for CX₃CL1 in the cross-talk between ECs and other cell types in the skeletal muscle. The present thesis shows that BM-derived cells incorporate into human skeletal muscle tissue both as ECs and fused with skeletal muscle fibers. Further, monocyte subsets display different temporal patterns in mobilization with exercise, and monocyte recruiting factors and endothelial adhesion molecules increase in skeletal muscle with exercise. MMPs increase with exercise with different responses to acute exercise versus a longer period of training. Data from this thesis support the idea that cross-talk between different cell types in the muscle tissue, including ECs, muscle fibers and macrophages, plays an important role in the adaptation of skeletal muscle to exercise.

To my family

LIST OF SCIENTIFIC PAPERS

The thesis is based on the following papers, referred to in the text by their Roman numerals:

- I. **Strömberg A**, Jansson M, Fischer H, Rullman E, Hägglund H, Gustafsson T. Bone marrow derived cells in adult skeletal muscle tissue in humans. *Skelet Muscle*. 2013 May 16; 3(1):12.
- II. **Strömberg A**, Rullman E, Jansson E, Gustafsson T. Exercise-induced upregulation of endothelial adhesion molecules in human skeletal muscle and number of circulating cells with remodeling properties. *J Appl Physiol*. 2017 May 1;122(5):1145-1154
- III. Rullman E, Norrbom J, **Strömberg A**, Wågsäter D, Rundqvist H, Haas T, Gustafsson T. Endurance exercise activates matrix metalloproteinases in human skeletal muscle. *J Appl Physiol*. 2009 Mar; 106(3):804-12.
- IV. **Strömberg A**, Olsson K, Dijksterhuis JP, Rullman E, Schulte G, Gustafsson T. CX3CL1– a macrophage chemoattractant induced by a single bout of exercise in human skeletal muscle. *Am J Physiol Regul Integr Comp Physiol*. 2016 Feb 1; 310(3):R297-304.

Publications by the author not included in the thesis:

Crossland H, Atherton PJ, **Strömberg A**, Gustafsson T, Timmons JA. A reverse genetics cell-based evaluation of genes linked to healthy human tissue age. *FASEB J*. 2017 Jan; 31(1):96-108.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BM	Bone marrow
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
DAPI	4', 6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
EC	Endothelial cell
ECM	Extracellular matrix
EPC	Endothelial progenitor cell
FBS	Fetal bovine serum
FCS	Fetal calf serum
FISH	Fluorescent <i>in situ</i> hybridization
GAPDH	Glyceraldehyde phosphate dehydrogenase
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule 1
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
PBS	Phosphate buffered saline
RNA	Ribonucleic acid
RT	Room temperature
SaC	Satellite cell
SD	Standard deviation
SSC	Saline sodium-citrate
THP-1	Human monocytic leukemia cell line
TIMP	Tissue inhibitor of metalloproteinase
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VO ₂ max	Maximal oxygen uptake

1 BACKGROUND

Tissue remodeling is a central process for all life. When a tissue is exposed to environmental stress such as injury, both local cells and cells from the periphery are recruited to regenerate and remodel the injured site. During the last decades, considerable research has been conducted to elucidate the biology of tissue stem cells and remodeling, aiming at understanding the mechanisms behind tissue regeneration and finding novel therapies for diseases.

The skeletal muscle tissue is composed of various cell-types and structures, including muscle fibres and blood vessels, and is a highly plastic organ; it has a great capacity of adapting to environmental demands even in the adult (126). Together with the accessibility of muscle tissue for *ex vivo* and *in vitro* analysis, this makes the skeletal muscle tissue ideal for remodeling studies. In the past, changes in skeletal muscle were assumed to be mainly related to mechanisms in the skeletal muscle fibers themselves. However, during the last decades, the importance of resident and circulating stem cells in the process of skeletal muscle remodeling has been largely recognized (35, 40). Also, all forms of tissue regeneration including that of skeletal muscle involve leukocytes. Current data suggest that leukocytes in the skeletal muscle aid by clearing cell debris and by releasing growth factors and chemokines, thus providing a microenvironment permissive of tissue repair and growth.

1.1 SKELETAL MUSCLE TISSUE STRUCTURE

Skeletal muscle tissue (**Fig 1**) is largely composed of long multi-nucleated muscle fibers containing actin and myosin filaments responsible for contraction. The fibers are post-mitotic and their maintenance is provided by the stem cell of skeletal muscle tissue, the satellite cell (SaC) (95). SaCs are quiescent cells located between the basal lamina and the cell membrane of the muscle fibers, the sarcolemma, and constitute ~2–3% of myonuclei in adult human skeletal muscle (148). When activated, by stimuli such as muscle injury or growth factors, SaCs start proliferating and are then called myoblasts. The myoblasts then differentiate and fuse together into myotubes or fuse directly with muscle fibers (40). In addition to SaCs, animal studies have suggested other sources of skeletal muscle stem cells, including hematopoietic cells (11, 137).

Each muscle fiber has its individual basal lamina which overlies the muscle fiber and its SaCs (127). It is composed of a collagen network containing laminins that anchor the SaCs. It also contains other extracellular matrix (ECM) proteins such as fibronectin and proteoglycans that bind inactive growth factors. The basal lamina is continuous with the endomysium, a complex connective tissue matrix which connects the fibers to each other (53).

An extensive capillary network surrounds each muscle fiber, which provides the fibers with oxygen and nutrients. The capillary density in skeletal muscle can be measured as capillaries per fiber or capillaries per area unit, where the latter depends on muscle fiber size. The skeletal muscle exhibits the capacity to increase the capillary density as an adaptation to exercise training (6). The generation of capillaries from pre-existing ones, angiogenesis, occurs through proliferation of endothelial cells (ECs), proteolytic degradation of the

extracellular matrix and migration of the ECs which then form a connection with an existing capillary (41).

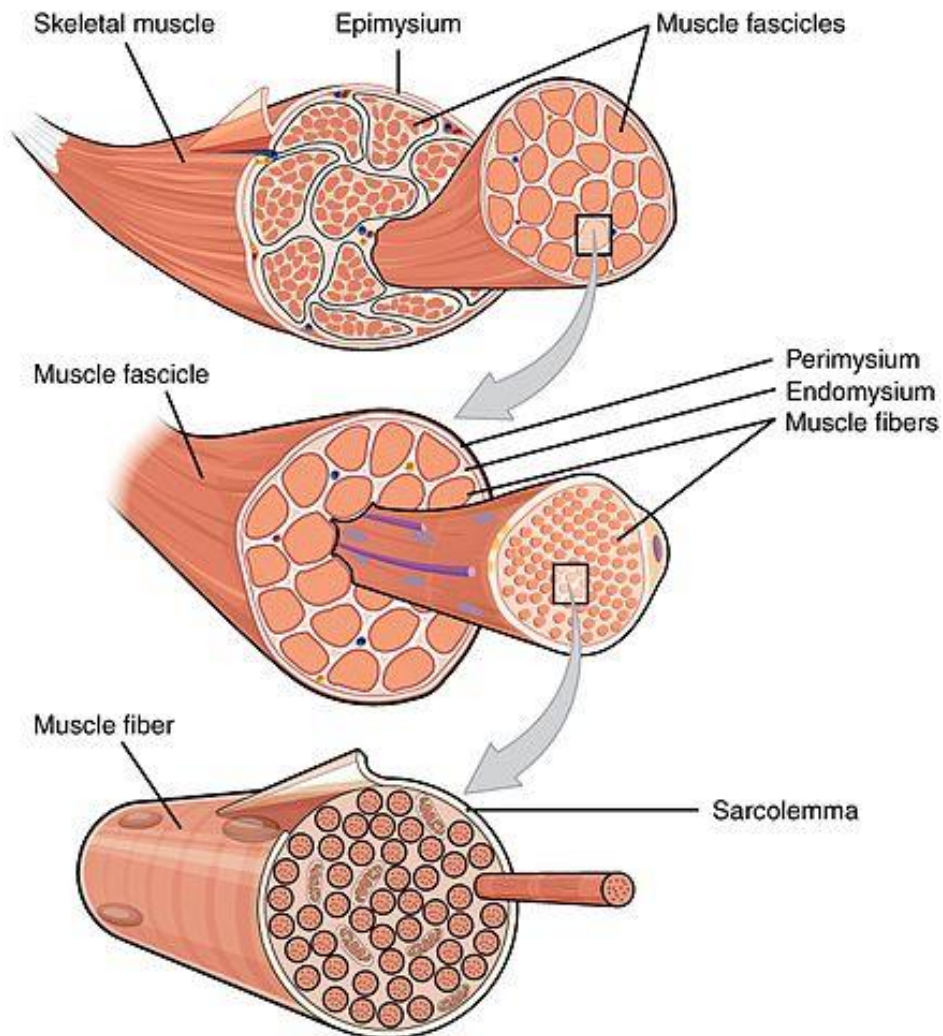


Figure 1. The skeletal muscle tissue consists of muscle fibers ensheathed by the muscle fiber membrane, the sarcolemma. The muscle fibers are surrounded by the endomysium, a thin layer of connective tissue.

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1.2 SKELETAL MUSCLE REMODELING

1.2.1 Conditions when skeletal muscle remodeling occurs

As mentioned above, the skeletal muscle tissue has a great capacity to adapt to the demands placed upon it, which is termed plasticity. Several different systems are involved in this adaptation, including the vascular, nervous, and immune system (43, 65, 118). Numerous diseases as well as aging affect the skeletal muscle tissue directly or indirectly, leading to altered structure and function. This includes neuromuscular disorders, inflammatory myopathies, heart failure and malignancies. In healthy individuals, the most important physiological stressor affecting the skeletal muscle is alteration of physical activity. Thus, exercise provides an ideal intervention to study the various aspects of skeletal muscle remodeling. Muscle adaptations with increased physical activity occur over a continuum and are dependent on the specific mode, duration, frequency and intensity of the exercise. Typically, endurance training, characterized by high-volume/low force muscle actions, leads to increased capillary formation and increased capacity for oxidative metabolism. On the contrary, resistance training with low-volume/high force actions, leads to increases in muscle mass and strength. It should also be acknowledged that the response to different exercise stimuli is highly variable across individuals, and thus, both high- and low-responders are evident both with endurance and resistance exercise.

1.2.2 Muscle capillarization

Regeneration and growth of tissues are dependent on the formation of new vessels (46). In the adult human, increased capillarization is rare under physiological circumstances, and can virtually only be seen in the female reproductive organs and in skeletal muscle tissue as a response to endurance-type training. With endurance training, the capillary density in skeletal muscle increases both in terms of capillaries per fiber and capillaries per area unit (6, 69, 136). This facilitates substrate exchange, oxygen delivery and decreases the oxygen diffusion distance (126). Different stimuli have been proposed to promote exercise-induced angiogenesis, including increased blood flow, mechanical stretch, lowered oxygen tension and metabolic perturbation (66, 136). Vascular endothelial growth factor A (VEGF-A) has been shown to be required for increased capillarization in skeletal muscle and, indeed, VEGF-A mRNA and protein levels are increased in skeletal muscle in response to endurance exercise (55, 108, 149).

1.2.3 Metabolism and mitochondrial biogenesis

Mitochondrial biogenesis – an increased mitochondrial content and enzyme activity – is well-established to occur in skeletal muscle with endurance exercise, and leads to a greater capacity for oxygen consumption and ATP synthesis (63). Endurance exercise also increases the amount of glucose and fatty acid transporters, together with an increased storage of glycogen and lipids in the skeletal muscle fibers. The increased mitochondrial content in combination with increased levels of oxidative enzymes yield a higher aerobic capacity of the skeletal muscle (34).

1.2.4 Extracellular matrix remodeling

Turnover of the ECM is required for angiogenesis, myotube formation, and reorganization of the matrix during muscle adaptation (53, 57). The skeletal muscle ECM protein turnover is influenced by physical activity, with increased expression of both collagens and non-collagenous ECM proteins and matrix metalloproteinases (MMPs) (61, 89, 100). MMPs are a family of enzymes that can digest individual components of the ECM in both physiological and pathological states (31). In humans, there are 23 known soluble and membrane-type MMPs. These enzymes are regulated both at the transcriptional level, through activation of precursor zymogens, and through inhibition by tissue inhibitors of metalloproteinases (TIMPs) (105). Members of the MMP family increase in skeletal muscle with exercise in animals (57, 83), and in humans a single endurance exercise bout increase the expression and activity of MMP-9 (124). Interestingly, MMP-9 degrades collagen IV which is one of the key components of the basal lamina of skeletal muscle fibers (127), and collagen IV is released from human skeletal muscle with a single bout of exercise (123). This indicates that basal lamina turnover occurs with a single endurance exercise bout and that MMP-9 is a plausible candidate behind the degradation of collagen IV with exercise. Other MMPs suggested to be important in skeletal muscle remodeling include MMP-2 and MMP-14 (57, 83).

1.2.5 Hypertrophy

Skeletal muscle hypertrophy is defined by increases in myofiber cross-sectional area and mass as well as myofibrillar protein content. Myofiber areas are analyzed through histological examination of skeletal muscle sections. Changes in whole-muscle size can be investigated through imaging techniques including magnetic resonance imaging, computerized tomography and ultrasound (47). An increased myofibrillar content reflects a net positive balance between protein synthesis and protein breakdown, which is achieved with both concentric and eccentric resistance exercise (117). Resistance training promotes hypertrophy of individual muscle fibers, which leads to increased muscle cross-sectional area and hence contractile force (139). Muscle hypertrophy can also be seen with endurance type training, albeit to a smaller extent (81).

1.2.6 Satellite cells

It is generally accepted that SaCs contribute to muscle fiber repair and growth. Data that indirectly show SaCs being important for muscle adaptation is the increase in SaC number with a single bout of resistance exercise and endurance exercise respectively (90, 97), where the eccentric bout of exercise also increased the number of SaCs progressing towards mitosis (97). Further, muscle hypertrophy with resistance training is paralleled by an increased fiber-type specific SaC content, and myonuclear number show correlative changes with increases in myofiber size (44, 74, 106). Interestingly, it has been suggested that the hypertrophic response to resistance training is positively correlated with the SaC content before the training period (115). However, it should be acknowledged that the requirement of myonuclear addition and SaCs in hypertrophy is still under debate, and that hypertrophy has been seen even without SaC contribution (96, 146). In addition to serving as a source of myonuclei to growing muscle fibers, SaCs also interact with neighboring cells by direct contact or by the release of soluble factors. For example, most SaCs are located in close

proximity to ECs in both human and animal skeletal muscle, and provide pro-angiogenic signals (32, 106).

1.3 BONE MARROW-DERIVED CELLS IMPLICATED IN SKELETAL MUSCLE REMODELING

While SaCs are considered essential for muscle regeneration (86, 104), efficient muscle recovery relies on many other cell types. Skeletal muscle tissue contains resident immune cells, and additional cells from the circulation can infiltrate the muscle tissue upon injury or increased mechanical stress. Whether other cell types than SaCs contribute to myonuclear addition to muscle fibers is debated, but it has been proposed that the SaC pool is supplemented with progenitor cells from other sources, such as the bone marrow (BM) (84, 141). An alternative route suggested for BM-derived cell contribution to skeletal muscle is via direct fusion to muscle fibers in response to a physiological stimulus such as injury (45, 54). In BM-transplanted mice, voluntary treadmill running increased the contribution of BM-derived cells to skeletal muscle fibers compared to mice that did not train, suggesting that endurance training is a sufficient stimulus to elicit contribution of BM cells to myofibers (110). Regardless of whether BM-derived cells actually contribute to cells in the skeletal muscle tissue, several reports from both animal and human have shown skeletal muscle infiltration of BM-derived cells with exercise and more so with damaging type exercise (36, 88, 94, 112). Thus, the importance for these cells in skeletal muscle remodeling may lie in their capacity to clear debris, secrete growth factors and regulate the inflammatory response. While different BM-derived cell subsets have been suggested to play a role in skeletal muscle regeneration and remodeling, the present thesis focuses on monocytes/macrophages and endothelial progenitor cells (EPCs).

1.3.1 Importance for monocytes/macrophages in muscle remodeling

In general, inflammatory processes in tissues occur in stages starting with the activation of resident macrophages and patrolling monocytes that in turn recruit neutrophils, which leads to a subsequent neutrophil-mediated monocyte recruitment from the circulation. After the initial pro-inflammatory phase, resolution of inflammation occurs through a shift towards a more anti-inflammatory environment. Finally, tissue repair and regeneration ensue, which include matrix remodeling and angiogenesis. In both *in vitro* studies, animal models and studies in humans, leukocytes in general and monocytes/macrophages in particular have been identified as essential for skeletal muscle remodeling primarily through pro-angiogenic and pro-mitotic signaling (7, 23, 24, 71, 99, 125, 129, 133). Monocyte depletion and selective inhibition of macrophage infiltration lead to severely impaired regeneration following muscle injury in mice (7, 129) which is characterized by fibrosis and delayed SaC proliferation (7). In steady state, macrophages in skeletal muscle primarily reside in the epimysium and perimysium. In injured mouse muscle, these resident macrophages are activated, migrate towards injured areas and recruit neutrophils and monocytes from the circulation via chemoattractants (21). In line with the phases of inflammation described above, it has been demonstrated that the monocytes recruited to damaged muscle are of a pro-inflammatory phenotype, phagocytose cellular debris and induce myoblast proliferation (7). These monocytes then differentiate to

macrophages and switch to an anti-inflammatory phenotype which promotes myoblast differentiation and angiogenesis (7, 71).

1.3.2 EPCs in skeletal muscle remodeling

In addition to angiogenesis, where vessels form through sprouting of resident ECs or intraluminal splitting, neovascularization has been proposed to occur also from circulating BM-derived EPCs that either contribute to endothelium in tissues or provide pro-angiogenic signaling to the resident ECs (9, 13, 77). Whether this occurs in human skeletal muscle with exercise is not known, but the circulating levels of these cells increase with exercise and EPCs collected from humans contribute to ECs in mouse ischemic muscle (1, 85, 120). In mice, EPCs have been found in capillaries in ischemic hindlimb muscle (8). Since exercise has been shown to increase the circulating level of EPCs and the level of pro-angiogenic factors in skeletal muscle, it can be speculated whether EPCs play a role in the formation of new capillaries with exercise.

1.4 MOBILIZATION OF CELLS INTO THE CIRCULATION

An increased number of leukocytes in the circulation is called leukocytosis, and occurs through mobilization of cells from the BM or marginal pools. Leukocytosis occurs in response to a variety of stressors including infection, inflammation, and physical activity (130). In humans, exercise increases the circulating level of BM-derived cell subsets suggested to contribute to skeletal muscle remodeling, including both monocytes and EPCs (85, 120). However, a difficulty with interpreting existing data regarding the circulating level of monocytes and EPCs is that different sets of markers and analysis procedures have been used to quantify them. Thus, there is still a lack of consensus regarding the change in number of circulating monocytes and EPCs with exercise in humans. The current nomenclature of human monocytes groups them in three subsets based on expression of CD14 and CD16; classical CD14⁺⁺CD16⁻, intermediate CD14⁺⁺CD16⁺ and nonclassical CD14⁺CD16⁺⁺ (155). Compared to classical monocytes, CD16 positive monocytes express higher levels of the receptors for endothelial adhesion molecules, which support them being capable of rapid binding to activated endothelium (154). The functional difference between these subpopulations is debated, and studies conducted to characterize monocytes support CD16 positive monocytes to be similar to macrophages and express pro-inflammatory cytokines, while others have shown that they are important for resolving inflammation (2, 103, 140).

Mechanisms proposed to regulate the augmented circulating cell level with exercise include shear stress from increased blood flow, increased plasma levels of various cytokines, and cortisol and catecholamines causing a release from the vessel walls (38, 48, 49). Already in the beginning of the 20th century it was shown that epinephrine caused leukocytosis in humans, and catecholamine-induced changes in leukocyte circulation have thereafter been examined in various studies (14). The fact that infusion of epinephrine in concentrations comparable to those seen in plasma with exercise mimics the exercise-induced changes in circulating leukocytes gives strong support for catecholamines playing a major role in the leukocytosis seen with physical activity (143). Plasma growth factors and chemokines shown to increase or correlate with the circulating number of monocytes and EPCs include VEGF-A, SDF-1, and G-CSF (25, 59, 60, 82, 102). However, with administration of the respective

factors, the peak increase in circulating cells is seen at least 1 day after administration (59, 60, 111). Thus, it can be questioned whether they are involved in the rapid mobilization reported with exercise.

1.5 RECRUITMENT INTO SKELETAL MUSCLE TISSUE

In human skeletal muscle, increased T-cells and macrophages located to epimysium and perimysium have been found after prolonged endurance exercise (94). With electrically stimulated isometric contractions in humans, known to induce myofiber protein damage, infiltration of macrophages was found in cytoplasm of myofibers (36, 88). With eccentric exercise, the peak of infiltrating macrophages was shown to occur between 4–7 days post exercise, while leukocytes started to accumulate already 3 h after the bout (112). Other studies showed no increase in leukocytes in the skeletal muscle tissue with eccentric exercise (92, 93). However, different exercise modes and leukocyte markers were utilized in the respective studies. Thus, the occurrence and time-course of muscular infiltration of leukocytes depends on the type, intensity and duration of exercise. In addition, large variation in individual responses to a given exercise bout has been reported.

The general paradigm of leukocyte recruitment from the circulation into tissues involves rolling and adhesion to endothelium and transendothelial migration, orchestrated by cell-adhesion molecules both on the surface of leukocytes and endothelium (144). However, whether endothelial expression of adhesion molecules increases in human skeletal muscle with exercise is not known. The mechanisms responsible for recruitment of monocytes and/or macrophages into the skeletal muscle tissue are just beginning to be discovered. In animal models of muscle injury, chemokines expressed in the muscle tissue have been shown to have an important role in recruitment of monocytes/macrophages and muscle regeneration (147, 153). Moreover, in a previous study investigating the interplay between human myogenic precursor cells and monocytes/macrophages, human myoblasts in culture were displayed to produce the chemokines CX₃CL1, CCL22 and CCL2, the angiogenic growth factor VEGF-A and the urokinase receptor uPAR, which selectively recruited monocytes (30). Further, these factors were displayed to be expressed by activated human SaCs *in vivo* and account for a large part of monocyte chemotaxis, suggesting them to be responsible for recruiting monocytes to human skeletal muscle. VEGF-A, in addition to SDF-1, is also demonstrated to have an important role in recruiting EPCs to angiogenic regions (67, 150) and data from our group and others have shown VEGF-A to increase in skeletal muscle both at the mRNA and protein level with a single bout of endurance exercise (55, 56, 121). The recruitment of EPCs by VEGF-A and SDF-1 is mediated partly through enhanced intercellular adhesion molecule 1 (ICAM-1) expression on ECs (67), and blockade of ICAM-1 significantly reduces recruitment of both EPCs and monocytes (131, 152). Other adhesion molecules suggested to be important for homing are E- and P-selectins and vascular adhesion molecule 1 (VCAM-1) (39, 87, 107). Another plausible mechanism behind how the number of leukocytes is increased in the skeletal muscle with exercise is the increased blood flow to the tissue. In rats, the accumulation of monocytes in a bacterial lesion showed a strong correlation with the blood flow to the lesion (70). Presumably, the increased blood flow together with the increase in adhesion molecules has additive effects on the leukocyte recruitment to the tissue.

The bioavailability of several factors suggested to be important for inflammatory cell recruitment, including VEGF-A, TNF-alpha, IL-1- β , IL8 and CCL2 is modulated by MMPs (15, 42, 51, 128). Thus, in addition to digesting ECM components creating paths for cell migration, MMPs regulate inflammatory cell recruitment through the cleavage of chemokines and cytokines (145). Of the different MMPs, MMP-2 and MMP-9 appear as two of the more important ones related to skeletal muscle function and dysfunction (26). MMP-9 level and activity is increased with a single endurance exercise bout in human skeletal muscle, without any change in MMP-2 (124). However, in injured mouse muscle, a study of MMP-2 and MMP-9 displayed different temporal expression of these proteins with MMP-9 being induced within 24 h after injury related to the early inflammatory phase, and MMP-2 displaying a slower increase related to fiber regeneration (79). Thus, it is plausible that a longer period of training displays the similar pattern of MMPs in human skeletal muscle.

2 SUMMARY AND AIMS

In mouse muscle, BM-derived cells have been displayed to contribute to muscle fibers and vessel growth, and endurance exercise has been shown to increase this contribution. Some studies have also shown that cells from the BM contribute to the SaC pool. However, whether this occurs in human is not known. BM-derived cells demonstrated to aid in muscle regeneration in human and animal models include monocytes/macrophages and EPCs. These cell types increase in the circulation with exercise, which has promoted the idea of their participation in skeletal muscle remodeling occurring with exercise. However, different protocols have been used for their quantification and regarding EPCs, no clear definition exists. For the cells to infiltrate the muscle tissue, they need to traverse the endothelial layer of vessels in the skeletal muscle. This involves the expression of chemokines and adhesion molecules on the endothelium. However, only little data exists regarding expression of such factors in skeletal muscle with exercise.

In the present thesis, the aims were:

- to investigate whether cells from the BM incorporate into skeletal muscle tissue in adult humans
- to measure the level of monocytes and EPCs, circulating cells suggested to be of importance for skeletal muscle remodeling, before and after an acute bout of exercise
- to analyze the expression of factors suggested to be important for cell recruitment in exercised skeletal muscle tissue, including:
 - i. endothelial adhesion molecules
 - ii. matrix metalloproteinases
 - iii. monocyte/macrophage recruiting factors

3 MATERIALS AND METHODS

3.1 EXPERIMENTAL DESIGN

Four studies were performed (**Table 1**). The experimental setup was explained to all subjects and their written informed consent was obtained before inclusion. All subjects completed a questionnaire, and in study II – IV only healthy, non-smoking subjects not taking any medication were included. The control subjects in study I fulfilled the same criteria. Each study was approved by the Regional Ethical Review Board in Stockholm and conformed to the declaration of Helsinki.

Table 1. Subject characteristics for the different studies. Data is presented as mean (SD).

Study	I	II	III	IV	
Subjects	5 women	20 men	10 men	6 women 11 men	3 men microdialysate
Age, yrs	34 (3)	26 (4)	24 (2)	25 (3) 26 (3)	23 (2)
Height, cm	167 (7)	181 (5)	180 (5)	171 (7) 182 (5)	182 (8)
Weight, kg	66 (10)	77 (6)	76 (9)	65 (7) 81 (7)	77 (7)
VO ₂ max ml * kg ⁻¹ * min ⁻¹	not tested	52 (6)	51 (6)	43 (4) 51 (5)	not tested

3.1.1 Study I

Three women transplanted with male hematopoietic stem cells 6 to 12 years previously participated in the study. Two healthy women served as controls. All patients were on post-transplant hormonal replacement therapy and in remission. Patient A had suffered from myelodysplastic syndrome, and was transplanted with BM from an unrelated donor. She had two children, a son and a daughter aged 11 and 13. Patient B had acute myeloid leukaemia, and had received BM-derived peripheral blood stem cells from her brother. Patient C had chronic lymphatic leukaemia, and was transplanted with BM from her brother. Neither patient B nor C had biological children. One of the control subjects had two sons aged 4 and 7, respectively, at the time of biopsy sampling. The other control subject had two children, a son and a daughter, aged 3 and 5. From all subjects, a muscle biopsy was obtained from the vastus lateralis muscle of one leg, and stored in -80°C until analysis. From the patients, one piece of the biopsy was stored for extraction of SaCs.

3.1.2 Study II

Twenty healthy male subjects were included in the study and divided in two groups: Group 1 and Group 2. Prior to the intervention, their maximal oxygen uptake (VO₂ max) was

determined using an incremental cycle ergometer test. On the experimental day, the subjects performed 60 min of cycling exercise. In Group 1, blood samples for flow-cytometric analysis of monocytes and EPCs and complete blood count were drawn pre and up to 2 h post exercise. In Group 2, muscle biopsy samples were obtained from the vastus lateralis muscle at rest and following exercise and analyzed for factors involved in recruiting circulating cells.

3.1.3 Study III

Ten healthy men were included in the study. Prior to the intervention, their VO_2 max was determined using an incremental cycle ergometer test. The subjects performed one-legged exercise four times per week for 5 weeks. Briefly, the subjects performed 45 min of one-legged exercise with a 20% restriction in blood flow at their highest tolerable work load. This was followed by exercise with the opposite leg without blood flow restriction at the same work load as the first leg. Hereby, both legs exercised with the same mechanical load, but with functional ischemia and increased metabolic stress in the restricted leg. Muscle biopsies were obtained before and 2 h after the first exercise bout, after 10 days of training, and after 5 weeks of training.

3.1.4 Study IV

Seventeen healthy subjects were included in the study and divided into an exercise group (8 men, 4 women) and a control group (3 men, 2 women). Prior to the intervention their VO_2 max was determined using an incremental cycle ergometer test. No significant differences were found between the groups regarding age, height, weight, or VO_2 max. The exercise group performed 60 min of cycling exercise, while the control group remained rested. Prior to cycling/rest, one biopsy was obtained from each leg, and then one biopsy was obtained at 30 min after, 2 h, 6 h and 24 h after exercise/rest. For the microdialysate part of the study, 3 healthy male subjects participated. Microdialysate was obtained from the vastus lateralis muscle of both legs at two different occasions with a sampling time of 4 h; one leg after exercise, the other leg without exercise. Four healthy subjects (20–31 years old, 2 women and 2 men) were recruited specifically for us to obtain muscle biopsies for isolation of primary human myoblasts.

3.2 EXERCISE PROTOCOLS

3.2.1 Cycling exercise – study II and IV

The subjects performed 60 min of cycling exercise on an electrodynamically loaded cycle ergometer. During the first 20 min, subjects cycled at 60 rpm at a work rate corresponding to 50 % of $\text{VO}_{2\text{max}}$, after which the work rate was increased to a work load corresponding to 65 % of $\text{VO}_{2\text{max}}$ for an additional 40 min. Ratings of perceived exertion was measured every 10 min using the 6-20 Borg scale (20). The subjects were allowed to drink water *ad libitum* during the experiment.

3.2.2 One-legged training – study III

One-legged training was performed 4 times per week for 5 weeks. One leg trained with restricted blood flow and the contralateral leg trained with non-restricted blood flow.

Restricted blood flow was achieved by local application of external pressure over the working leg. Briefly, the subjects were positioned supine with both legs in a pressure chamber, with one foot strapped to the pedal of a cycle ergometer. The chamber opening was sealed off at the level of the crotch by a rubber diaphragm with holes for the legs. The pressure in the chamber was elevated to 50 mmHg, which reduced the blood flow to the leg with ~20 %. Each session started by 45 min of training with the restricted leg. The subject was instructed to cycle at a constant pedaling rate of 60 rpm at the highest tolerable work load that could be sustained at any given moment, taking into account that the whole 45 min session must be completed. After 10 min of rest, the same workload protocol was performed by the other leg, but with normal atmospheric pressure in the chamber. Thus, the two legs performed the same power in each session.

3.2.3 One-legged exercise – study IV

The subjects performed dynamic constant load one-legged knee extension exercise (60 rpm) in the sitting position using a modified cycle ergometer using the dominant leg (5). One week prior to the experiment, subjects performed a one-legged incremental exercise test: after 1 min at 60 rpm and 25 W, workload was increased with 5 W every minute until exhaustion. Subjects were instructed to refrain from exhaustive exercise during the week prior to the experiment. On the day of the experiment, initial workload was 60% of maximal workload. After 20 and 40 min, workload was adjusted on the basis of effort assessed with the Borg scale.

3.3 EXPERIMENTAL PROCEDURES

3.3.1 VO₂ max

The VO₂ max was determined using an incremental cycle ergometer test until exhaustion, with respiratory gases continuously analyzed (Sensor Medics Vmax 229, Intra Medic AB, Sweden). Before the test, electrocardiography was performed to exclude pathologies.

3.3.2 Muscle biopsy sampling

In all studies, muscle biopsy samples from the vastus lateralis muscle were obtained using the percutaneous needle biopsy technique (16). Following local anesthesia of the skin and fascia using Carbocaine (10 mg/ml), a biopsy was obtained from the middle part of the vastus lateralis muscle using a Bergström-needle with suction applied. The biopsies were cleaned from any visible fat and then frozen within 20 s in 2-methylbutane cooled to freezing by liquid nitrogen and stored at -80°C until analysis.

3.3.3 Satellite cell extraction and culture

For extraction of SaCs, muscle biopsy samples were put in sterile phosphate buffered saline (PBS) with 1% penicillin-streptomycin and stored overnight at 4°C. After ~20 h, the biopsy was digested in 0.25% trypsin EDTA in 37°C, 5% CO₂ with gentle agitation for 20 min. Undigested tissue was allowed to settle for 5 min and the supernatant was collected in DMEM-F12, with 20% FCS and 1% penicillin streptomycin. The cells were cultured in flasks until reaching ~70% confluency, when used for myoblast experiments. For myotube

experiments, a low serum medium was used to induce differentiation of myoblasts into myotubes.

3.3.4 Immunohistochemistry

Mounted muscle biopsies were cut into sections (4 or 5 μm thick) and placed on Superfrost/Plus microscope slides (Thermo Fischer Scientific). The sections were fixed in either 4% phosphate buffered formaldehyde, -20°C acetone or 99% ethanol. The tissue sections were blocked in 4% FBS/PBS for 30 min, and then incubated with the primary antibody overnight at 4°C in a humid chamber. The following day, sections were incubated with secondary antibodies for 60 min in room temperature, and mounted in ProLong Gold antifade reagent (Life Technologies, Carlsbad, CA) containing the DNA stain DAPI. A Leica DMLA microscope equipped with a Leica DFC 450 digital camera (Leica Microsystems AB, Sweden) was used to obtain conventional fluorescent images.

3.3.5 Fluorescent *in situ* hybridization (FISH)

Fluorescent *in situ* hybridization of sex chromosomes was performed after antibody staining of muscle sections or cytopun myoblasts. The glass slides were put in $2 \times$ saline sodium-citrate (SSC) buffer, and the cover slips were allowed to fall off. The chromosome enumeration probes (CEP) for the X and Y chromosomes (Vysis CEP X (DXZ1) and CEP Y (DYZ1)), were mixed with hybridization buffer according to the manufacturer (Abbott-Vysis Inc Downers Grove, IL, USA); 1.5 μL of the probe solution was added to a round cover slip (Thermo Fischer Scientific), and the glass slide was then put on top of the cover slip. Glass slides and probe mixture were denatured together in a Vysis HYBrite (Abbot Diagnostics) for 20 h. When the hybridization was complete, the slides were immersed in $2 \times$ SSC until the cover slips fell off. The slides were then washed and subsequently allowed to air-dry in the dark before mounting in VectaShield antifade (Vector Laboratories) with DAPI or propidium iodide as nuclear staining.

3.3.6 RNA extraction and mRNA quantification

Total RNA from skeletal muscle, human umbilical vein endothelial cells (HUVECs), THP-1 cells (human monocytic cell line), primary human myoblasts and myotubes was prepared by the acid phenol method using TRIzol. About 20 mg of muscle tissue was homogenized in TRIzol with a polytron, while for cultured cells all media was removed and TRIzol added directly to the culture plates. The RNA was quantified by measuring absorbance at 260 nm using NanoDrop 2000 (Thermo Scientific, Göteborg, Sweden). RNA was reverse transcribed into cDNA using the high-capacity cDNA reverse transcription kit (Life Technologies). TaqMan gene expression assays were purchased from Life Technologies, and GAPDH was used as an endogenous control. Gene expression levels were determined using the $2^{-\Delta\text{CT}}$ method, relating mRNA changes as a ratio to the reference gene.

3.3.7 HUVEC stimulation with VEGF-A or IL6

HUVECs (#C-003-5C, Life technologies) were cultivated in M200 medium (Life technologies) with 20 % fetal bovine serum (FBS). At passages 2–3, the HUVECs were stimulated for 2 h with either serum-free M200 medium (control) or recombinant protein

diluted in serum-free M200 medium. The proteins used were VEGF-A and IL-6 (R&D systems) at concentrations of 100 ng/ml.

3.3.8 Laser capture microdissection

Briefly, 8 µm muscle sections placed on glass slides were fixed in -20 °C acetone, and then washed with PBS containing RNase inhibitor (50 U/100 I, SUPERase, Ambion). The slides were dehydrated and then microdissection was performed using the P.A.L.M system (Carl Zeiss microimaging). Individual muscle fibers and corresponding areas of muscle tissue covering all cell types were dissected into caps. Total RNA was isolated using the PicoPure RNA isolation kit (Arcturus Engineering) according to the manufacturer's description.

3.3.9 Zymography

Zymography supplies were purchased from Invitrogen. In brief, muscle protein homogenate were diluted in $2 \times$ Tris-glycine SDS buffer and electrophoretically separated under non-reducing conditions. The gels were incubated overnight at 37 °C in developing buffer. After staining with Coomassie blue and destaining for 2 days with acetic acid and methanol in water, gelatinase activity was evident by clear bands against a dark blue background. Quantification was performed using the densitometry software Fujifilm Image gauge version 3.46.

3.3.10 Microdialysate procedure and HUVEC stimulation

Microdialysate was obtained from the vastus lateralis muscle after one-legged exercise or rest on two separate occasions using CMA 71 high cut-off brain microdialysis catheters with a cut-off of 100 kDa (CMA Microdialysis AB, Sweden). After local anesthesia, the catheter was inserted with a guided cannula and perfused with Ringer's acetate at a rate of 2 µl/ min. During the 4 h sampling, the subjects rested in a supine position. The dialysate was collected in vials that were switched every 30 min, and then the collected sample was transferred to -80°C. During the first 30 min after insertion, the catheters were infused at a rate of 5 µl/ min. The samples collected during the first 60 min post insertion were not used for the stimulation assay. HUVECs (C-003-5C; Life Technologies) were cultured in 24-well plates in Medium 200 supplemented with low-serum growth supplement, and 1% antibiotic-antimycotic (AbAm) (Life Technologies). When confluent, the cells were stimulated for 2 h with microdialysate diluted 1:2 in Ringer's acetate. Two wells were used per condition (resting leg, exercised leg), which were pooled in TRIzol (Life Technologies) at cell harvest prior to RNA extraction.

3.3.11 Stimulation of THP-1 cells, primary human myoblasts and myotubes with CX₃CL1.

THP-1 monocytic cells (Sigma-Aldrich, St. Louis, MO) were cultured in RPMI 1640 medium GlutaMAX (Life Technologies) supplemented with 1% AbAm, 10% FBS, 1 mM sodium pyruvate, and 0.05 mM 2-mercaptoethanol. For the stimulation assay, cells were suspended at 1×10^6 cells/ml in serum-free RPMI medium, and 2 ml were plated in each well in a six-well plate, with 0, 10, or 100 ng/ml recombinant human CX₃CL1 (R&D Systems, Minneapolis, MN). After isolation of SaCs, growth medium was changed every 3rd or 4th

day until cells reached 60% confluence. The myoblasts then were trypsinized and seeded in six-well plates at a density of 80,000 cells per well, in proliferation medium. All cultures had desmin positivity >90% analyzed using cytocentrifuged cells. After 2 days, the medium was changed to low-serum medium (DMEM/F-12 containing 2% FBS, 1% AbAm), and the cells were stimulated with 0, 10, or 100 ng/ml of CX₃CL1 for 5 h, with two wells per condition. For myotube experiments, myoblasts in six-well plates were allowed to reach 90% confluence, and then the medium was changed to a low-serum medium. After 3 days, the myotubes were stimulated with CX₃CL1 using the same protocol as for the myoblasts. After stimulation, the cells from each condition were pooled in 1 ml of TRIzol, and RNA extraction was performed.

3.3.12 ELISA – muscle and plasma

Frozen muscle biopsies were homogenized in 0.1 M potassium phosphate buffer containing 0.05% BSA and cOmplete protease inhibitor cocktail tablets (F. Hoffmann-La Roche). Homogenization was performed on ice using glass homogenizers, and the homogenates then were rotated for 1 h at 4°C. The homogenates were then centrifuged for 10 min at 1,500 g in 4°C, and subsequently stored at -80°C until analysis. The protein concentration was measured using the Bradford assay. The muscle homogenate was analyzed for CX₃CL1 protein concentration by ELISA following the manufacturer's instructions (R&D Systems).

Blood samples were centrifuged at 1,000 g for 10 min. An additional centrifugation step of the plasma at 10,000 g for 10 min at 4°C was included to remove platelets. The samples were immediately frozen in liquid nitrogen, and stored at -70°C until further analysis. The plasma concentrations of SDF-1 alpha, VEGF-A and G-CSF were determined using a sandwich enzyme-linked immunoassay (R&D Systems Inc) according to the manufacturer's directions.

3.3.13 Immunostaining of blood cells

All antibodies, the TruCount tubes and the lysis buffer were from BD Biosciences (San Jose, California, USA). The TruCount tubes contain fluorescent beads which allows for determining the absolute cell number in blood. Antibodies were added to the tubes without touching the metal grid in the bottom of the tubes. Thereafter, 50 µl of blood were added to each tube by reverse pipetting. The tubes were incubated for 20 min at room temperature in the dark before 450 µl of 1 × lysis solution (no. 555899) was added to each tube. The tubes were incubated for 10 min at room temperature in the dark and then protected from light and analyzed within 1 h.

3.3.14 Flow cytometry

Sample acquisition was performed on a FACS Aria cell sorter with FACSDiva 6.1 software (BD Biosciences) with a flow rate of < 10,000 events per second. Fluorescence compensation was performed with anti-mouse Igk CompBeads (BD Biosciences, no. 552843) and the respective antibodies. The bead number was obtained from an ungated CD16/CD45 plot for the analysis of MO subsets, and an ungated CD34/CD45 plot for the analysis of EPCs. The bead number was used to calculate the absolute cell number per volume unit of blood.

3.3.15 Confocal microscopy

In study I, tissue sections were visualized with a Zeiss LSM710 confocal system using a Plan-Apochromat 63×/1.40 oil objective. Z-stacks were acquired sequentially using a 405 diode laser and the 488 laser line of the Argon laser. To determine whether a Y chromosome belonged to a muscle fiber nucleus, Y chromosome staining and DAPI nuclear staining had to colocalize and the nucleus of interest had to be on the same focal plane as the muscle membrane staining. Z-stacks were presented as maximum intensity projections. Three-dimensional rendering, maximum intensity projections and deconvolution were done with the AutoQuant X3 software.

In study II, fluorescent signals were detected with the same Zeiss LSM710 confocal system in combination with the ZEN2011 software (Zeiss, Jena, Germany) using the laser lines 405 (DAPI), 488 (Alexa Fluor 488) and 561 (Alexa Fluor 568) for fluorescence excitation. Image rendering was done with Bitplane Imaris software using the volume tool.

3.3.16 Statistics

Study II - Data for monocyte and EPC numbers, plasma protein and catecholamine levels were analyzed with one-way ANOVA for repeated measures. The Holm-Sidak test for all pairwise comparison was used as post hoc analysis. A power analysis was performed for the EPC data using G*Power version 3.1.9.2 to determine the subject number required to obtain a significant effect of exercise. The data for plasma VEGF-A was not normally distributed and therefore the Friedman test was used. To ensure normal distribution, logarithmic transformation to the base 10 was performed on mRNA data from skeletal muscle and HUVECs. The mRNA data was then analyzed with the Student's *t*-test.

Study III – The data were analyzed using logarithmic-transformed ratios for mRNA and logarithmic-transformed densitometric values for zymographies. A two-way ANOVA for repeated measures was used to evaluate the effects of training in the two exercise conditions (R + UR) on mRNA and protein content. Planned comparison was used to locate differences corresponding to significant interactions or when no interaction was found to locate differences corresponding to significant main effects in the ANOVA models. Due to high variance, a non-parametrical analysis (Wilcoxon matched-pair test) was performed on the MMP-9 data in addition to the ANOVA. In the figures, samples are expressed relative to the corresponding trial control sample (pre-exercise), which was set at 1.

Study IV - The effect of exercise on gene and protein levels in human skeletal muscle was analyzed using two-way ANOVA for repeated measures. The Holm-Sidak test for all pairwise comparison was used to locate the points of interaction. The effect of CX₃CL1 stimulation on gene expression in myoblasts, myotubes, and THP-1 cells was analyzed using one-way ANOVA for repeated measures and the Holm-Sidak post hoc test. Gene expression in HUVECs was analyzed using the Student's *t*-test.

In all studies, differences were considered statistically significant at $P < 0.05$.

4 RESULTS

4.1 BONE MARROW-DERIVED CELLS IN HUMAN SKELETAL MUSCLE

Y chromosomes were found in muscle fiber nuclei (**Fig 2**) and in EC nuclei in skeletal muscle of women transplanted with male BM. No Y chromosomes were detected in CD56+ SaCs in the tissue sections analyzed. For subject A and B, 0.6% of the skeletal muscle fibers counted contained a Y chromosome within a nucleus. In subject C, 0.7% of the fibers had a Y chromosome inside a nucleus. For ECs, some of the cells were cut so that the nucleus was not visible but the cells were positive for CD31. For subject A, 0.6% of the ECs contained a Y chromosome (0.9% per EC with visual DAPI staining). For subject B, 0.3% (0.5% of ECs with DAPI) of ECs had a Y chromosome. Subject C had 0.4% Y chromosome containing ECs (0.6% of ECs with DAPI). In muscle sections from the skeletal muscle biopsies retrieved from healthy female controls, no Y chromosomes could be detected.

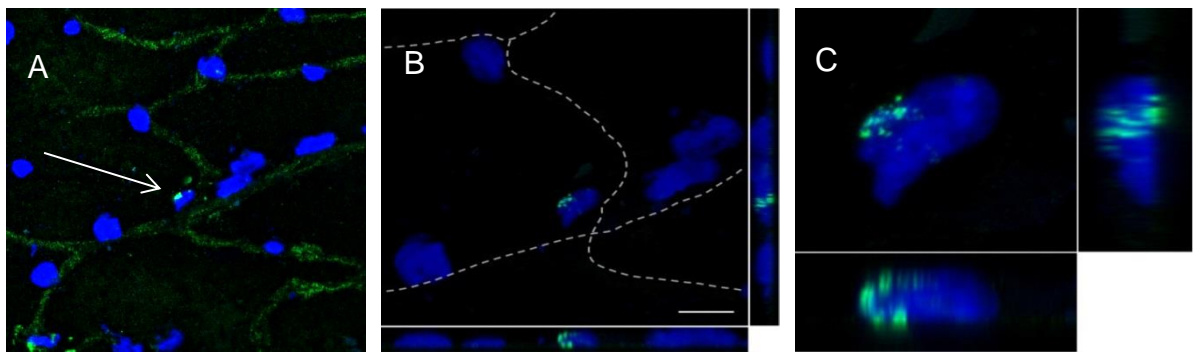


Figure 2. Y chromosome-positive nuclei are incorporated into host muscle fibers. Microphotographs show confocal images of combined FISH (Y chromosome, bright green, arrow) and immunofluorescence for caveolin-3 (green). Nuclei were counterstained with DAPI. **(A)** Microphotograph depicting a single Y chromosome-positive donor nucleus (arrow). Caveolin-3 staining is used to visualize muscle fiber membranes. **(B)** provides a Z-stack of the muscle fiber in **(A)**. Dashed lines mark muscle fiber membranes indicating that the Y chromosome nucleus is localized inside the muscle fiber. Size bar = 10 μ m. **(C)** A high magnification Z-stack of the double-stained nucleus depicted in **(B)** clearly shows the integration of the Y chromosome DNA in the nucleus.

SaCs were extracted from the biopsies obtained from the women transplanted with male bone marrow. About 80% of the cytocentrifuged cells from the SaC extraction were positive for desmin. None of the cells analyzed contained a Y-chromosome, indicating that bone marrow derived cells do not give rise to SaCs (**Fig 3**).

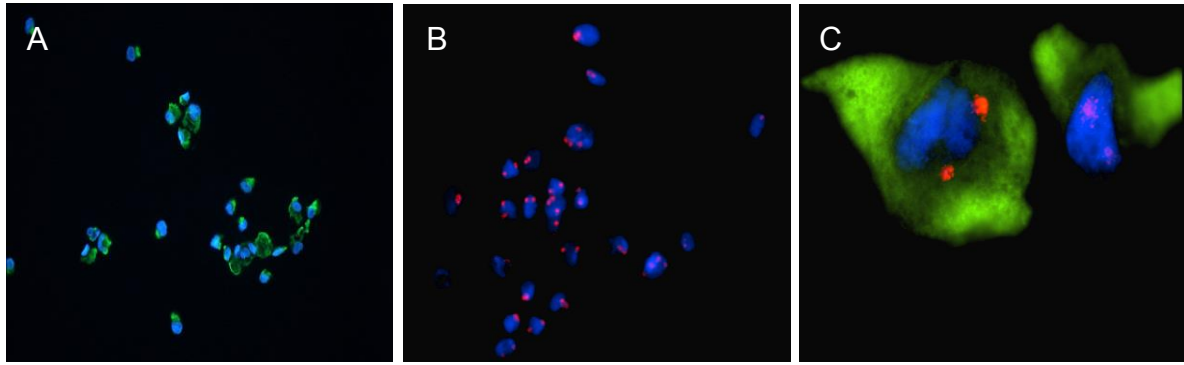


Figure 3. Immunofluorescent and FISH analysis of cytocentrifuged myoblasts from satellite cell extraction. (A) Desmin staining (green) before XY-FISH, 20 × magnification. (B) Myoblasts after XY-FISH. X chromosomes were stained red and Y chromosomes green. All cells were negative for the Y chromosome, 20 × magnification. (C) Myoblasts stained with desmin (green) containing two X chromosomes (red), 100 × magnification. The nuclei were counterstained with DAPI.

4.2 LEVELS OF MONOCYTES AND EPCS WITH EXERCISE

The number of classical $CD14^{++}CD16^{-}$ monocytes with exercise changed in a biphasic pattern with a significant increase directly after exercise and also at 2 h after exercise. The intermediate $CD14^{++}CD16^{+}$ monocytes and non-classical $CD14^{+}CD16^{++}$ monocytes were significantly increased directly after exercise. $CD45^{dim}CD34^{+}VEGFR2^{+}$ cells did not change significantly with exercise ($P = 0.08$). The power analysis performed utilizing the data obtained for both $CD45^{dim}CD34^{+}$ and $CD45^{dim}CD34^{+}VEGFR2^{+}$ cells displayed that with 14 subjects there would have been a significant effect on EPC number with exercise with a power > 0.80 and alpha-level 0.05. For cell numbers see **Table 2**.

4.3 LEVELS OF PROTEINS AND CATECHOLAMINES IN PLASMA WITH EXERCISE

The plasma level of G-CSF protein was significantly increased 2 h post exercise without adjustment to albumin. When adjusted to the plasma albumin concentration, the G-CSF level was not significantly elevated after exercise ($P = 0.09$). Similar to G-CSF, following adjustment to plasma albumin, the concentration of SDF-1 did not change in response to exercise. Exercise did not change the plasma level of VEGF-A regardless of adjustment to albumin. The plasma levels of both epinephrine and norepinephrine were significantly increased directly post exercise and restored to resting levels at post 30. For levels of proteins and catecholamines, see **Table 3**.

Table 2. Number of monocytes and EPCs in blood before and after exercise.

	Pre	Post	Post 30	Post 2 h
Monocytes (nr × μl ⁻¹)				
CD14 ⁺⁺ CD16 ⁻	373 (111)	574 (154) *	417 (132)	653 (199) *
CD14 ⁺⁺ CD16 ⁺	11 (8)	20 (20) †	12 (18)	14 (19)
CD14 ⁺ CD16 ⁺⁺	27 (8)	51 (19) †	23 (12)	33 (14)
EPCs (nr × μl ⁻¹)				
CD45 ^{dim} CD34 ⁺	1.4 (0.7)	2.7 (1.5)	2.2 (2.3)	2.9 (2.2)
VEGFR2 ⁺	0.3 (0.2)	1.0 (1.2)	1.2 (2.4)	1.8 (2.2)

Values are means (SD) and the cell numbers adjusted for hemoconcentration. EPCs are displayed as the total number of CD45^{dim}CD34⁺ cells, and the number of these positive for VEGFR2. * = significantly different from pre and post 30, $P < 0.001$. † = significantly different from all other time points, $P < 0.05$.

Table 3. Levels of proteins and catecholamines in plasma before and after exercise.

		Pre	Post	Post 30	Post 2 h
G-CSF	pg × ml ⁻¹	14 (8)	16 (7)	14 (8)	18 (8)
SDF-1	pg × ml ⁻¹	2058 (247)	2089 (204)	1980 (188)	2037 (203)
VEGF-A	pg × ml ⁻¹	21 (17)	24 (20)	21 (6)	21 (13)
Epinephrine	nmol × l ⁻¹	0.2 (0.1)	0.9 (0.8) *	0.2 (0.1)	0.2 (0.1)
Norepinephrine	nmol × l ⁻¹	1.6 (0.8)	10.8 (7.4) †	1.7 (0.6)	1.9 (0.7)

Values are means (SD) and adjusted for hemoconcentration. * = significantly different from all other time points, $P < 0.01$. † = significantly different from all other time points, $P = 0.001$.

4.4 SKELETAL MUSCLE GENE AND PROTEIN EXPRESSION WITH EXERCISE

4.4.1 Study II

The skeletal muscle tissue mRNA levels of ICAM-1, VCAM-1 and E-selectin were significantly upregulated 2 h after exercise. However, the mRNA level of E-selectin was extremely low. IL-6 and VEGF-A mRNA levels were significantly increased 2 h after exercise. The SDF-1 mRNA level and the expression of the reference gene GAPDH did not change with exercise. Double staining for ICAM-1, VCAM-1 or E-selectin and the endothelial marker CD31 showed that ICAM-1, VCAM-1 and E-selectin were localized to the endothelium (**Fig 4A-I**).

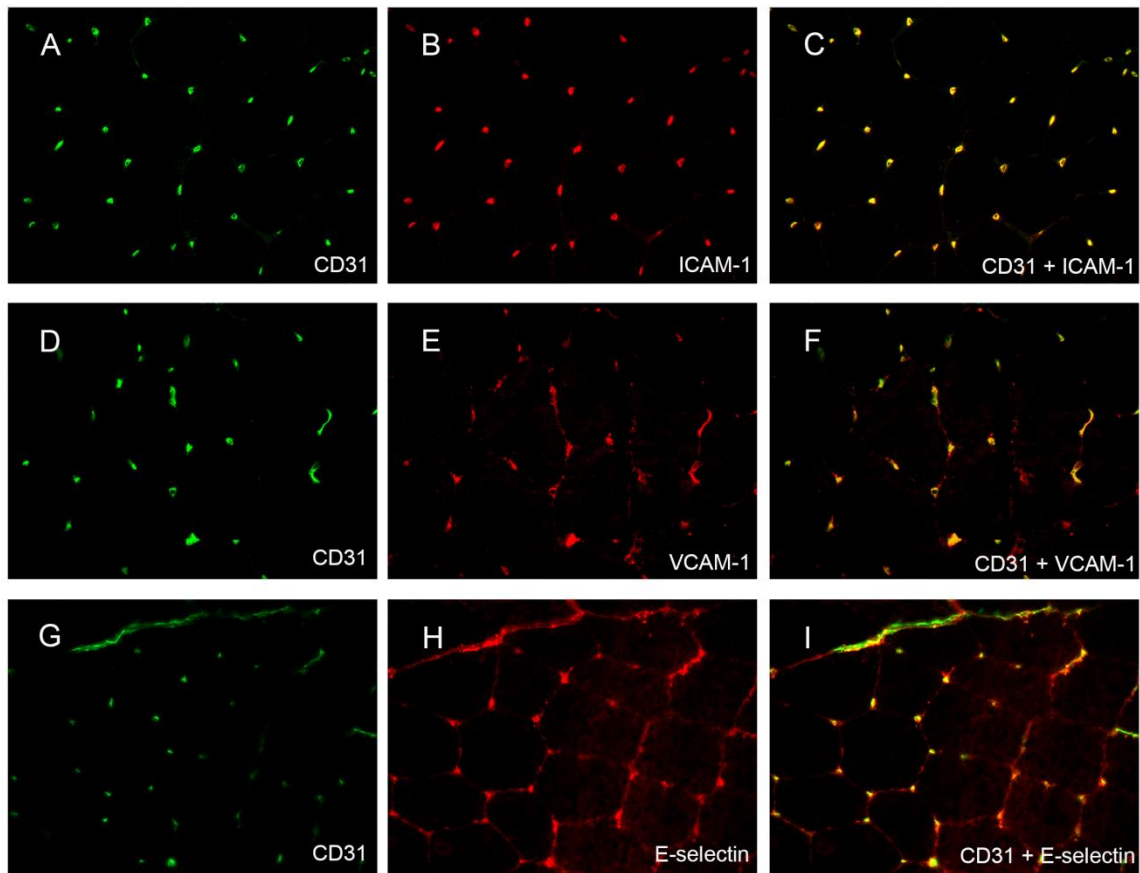


Figure 4. Endothelial adhesion molecules in skeletal muscle tissue. Representative captions (20 x magnification) of skeletal muscle sections with immunofluorescent staining for ICAM-1 (**B**), VCAM-1 (**E**) and E-selectin (**H**). The images show that the adhesion molecules are expressed on endothelial cells stained for CD31 (left panels; **A**, **D**, **G**). Images at the right (**C**, **F**, **I**) display overlays.

4.4.2 Study III

The skeletal muscle tissue mRNA level of MMP-9 was increased with a single bout of exercise in both the restricted (R) and unrestricted (UR) condition. No changes were seen for MMP-2, MMP-14 or TIMP-1 mRNA after a single bout of exercise. The mRNA levels of MMP-2, MMP-14 and TIMP-1 were all higher after 10 days of training and remained elevated after 5 weeks of training, independent of training condition (no interaction between training and condition). No change was seen for MMP-9. Laser capture microdissection (**Fig 5**) showed MMP-2 to be expressed by muscle fibers, while no MMP-9 mRNA could be detected in the dissected fibers.

MMP-9 protein (pro-MMP-9 + active MMP-9) was increased after a single bout of exercise (both UR + R), while the level of MMP-2 did not change (**Fig 6**). MMP-2 protein was higher after 10 days training and remained elevated at 5 weeks (both UR + R) while no change was seen for MMP-9.

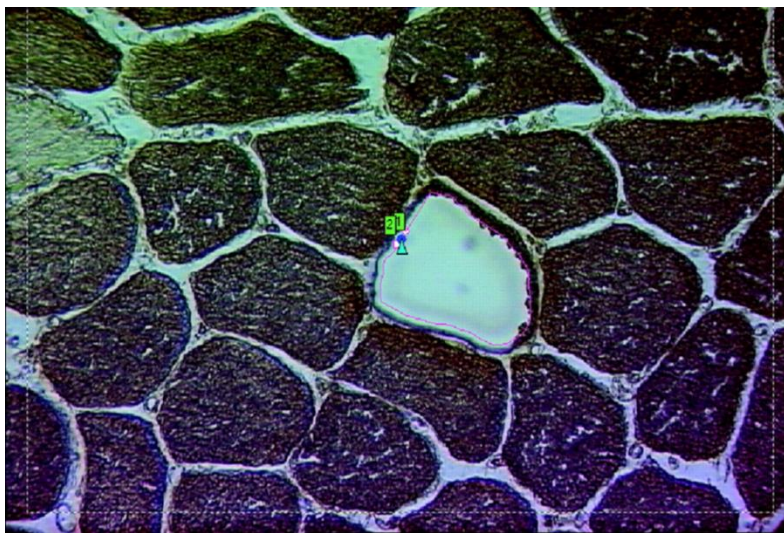


Figure 5. Laser capture-microdissected muscle fiber. The circle indicates a muscle fiber that has been microdissected out for mRNA analysis. Altogether, 100 fibers were isolated from each section. Fibers were microdissected with a clear margin to the cell boundaries to avoid contamination from adjacent cells. Arrows and numbers come from laser-capture software.

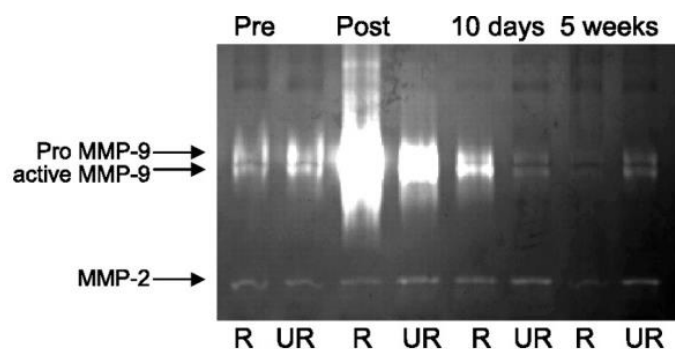


Figure 6. Illustration of a representative zymogram. MMP, matrix metalloproteinases; Pre, sample obtained before exercise; Post, sample obtained 2 h after the first exercise bout; 10 days and 5 weeks of exercise training, respectively; R and UR, sample obtained from the leg exercised with restricted and unrestricted blood flow respectively.

4.4.3 Study IV

CX₃CL1mRNA increased in the skeletal muscle tissue at 30 min post exercise (**Fig 7 A**). No change was seen in the control group (interaction between exercise and control $P = 0.002$). For the CCL2 and CCL22 mRNA levels (**Fig 7 C and D**), no interaction was found between the groups. There was no difference between men vs. women in the mRNA levels of CX₃CL1, CCL2, or CCL22. Since no changes were seen between exercise and rest in the gene expression of CCL2 and CCL22, the remainder of the study focused on CX₃CL1. The protein level of CX₃CL1 in the skeletal muscle homogenate was increased at 2 h post exercise (**Fig 7 B**). No change in CX₃CL1 protein levels was seen in the control group (interaction between exercise and control, $P = 0.04$). Confocal imaging displayed CX₃CL1 to colocalize with the endothelial cell marker CD31. SaCs showed no or very weak CX₃CL1 staining.

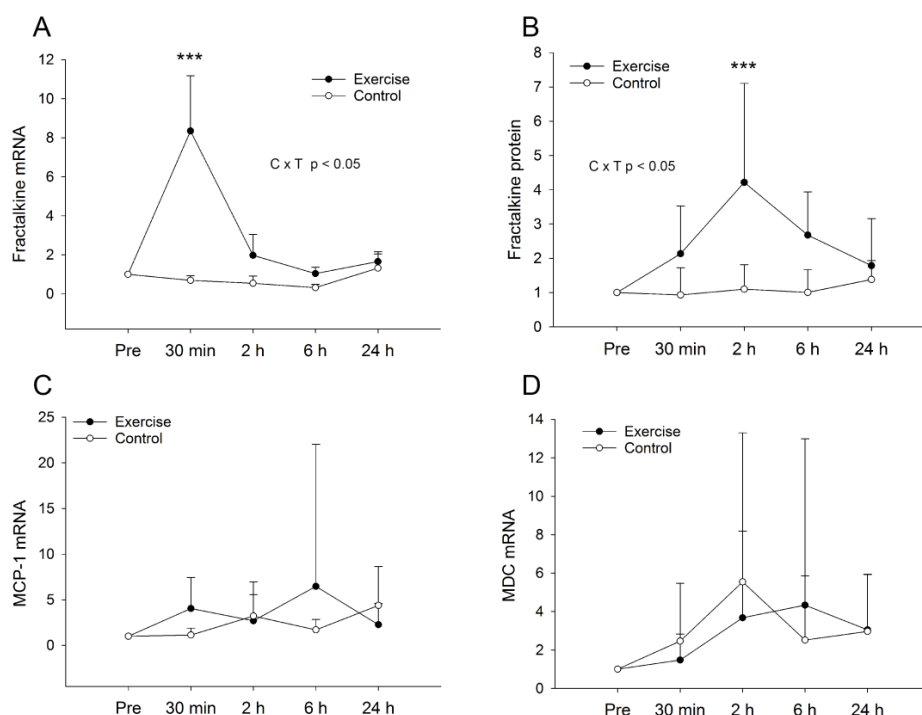


Figure 7. Fold change in skeletal muscle expression of CX₃CL1, CCL2 and CCL22 with exercise or rest. The mRNA level (A) and protein level (B) of CX₃CL1 increased with exercise while no change was seen in the control group. No interaction was seen between exercise and control groups regarding mRNA levels of CCL2 (C) and CCL22 (D). C x T, a significant interaction between condition (C) and time (T), $P < 0.05$. *** = $P < 0.001$ vs Pre.

4.5 GENE EXPRESSION IN STIMULATED HUVECS, MONOCYTES, MYOBLASTS AND MYOTUBES

4.5.1 Study II

To evaluate if EC adhesion molecules are induced by factors known to increase in skeletal muscle with exercise, HUVECs were stimulated with VEGF-A and IL-6. VEGF-A stimulation significantly increased the mRNA levels of VCAM-1 and E-selectin compared with control while ICAM-1 mRNA levels did not change. Stimulation with IL-6 for 2 h did not significantly increase the level of ICAM-1, VCAM-1 nor E-selectin.

4.5.2 Study IV

To investigate whether factors in the skeletal muscle tissue fluid could induce the expression of CX₃CL1 in ECs, HUVECs were stimulated with microdialysate obtained from exercised muscle. The mRNA level of CX₃CL1 increased 1.9-fold in HUVECs stimulated with exercise microdialysate compared with microdialysate from the resting leg, suggesting that at least part of the induction in CX₃CL1 may be due to factors increasing in the muscle interstitial fluid with exercise.

THP-1 monocytic cells were stimulated with CX₃CL1 to assess whether CX₃CL1 could modulate their inflammatory profile (**Fig 8 A**). The expression of proinflammatory TNF- α and IL-6 and anti-inflammatory IL-10 were increased significantly in cells treated with 100 ng/ml CX₃CL1. For MMP9 and TGF- β , the ANOVA detected a significant treatment effect, which, however, could not be located with the post hoc test. To further investigate plausible biological effects of CX₃CL1, the expression of factors involved in muscle remodeling was measured in primary human myoblasts (**Fig 8 B**) and myotubes (**Fig 8 C**) following CX₃CL1 stimulation. In myoblasts, the expression of IL-6 was increased in cells treated with 100 ng/ml of CX₃CL1, and there was a trend toward increased CCL2 and IL-8. 100 ng/ml CX₃CL1 decreased the level of MyoD, but it did not alter myogenin or VEGF-A. Myostatin and MMP9 exhibited a tendency to increase, although MMP9 was virtually not expressed, with an average CT value of 35.4. Myotubes increased their expression of IL-8, IL-6, MMP9, and CCL2 at 100 ng/ml, while no change was seen for VEGF-A, myostatin, MyoD, or myogenin. Because IL-8 was highly induced in myotubes with CX₃CL1 treatment, immunofluorescent staining of IL-8 was performed in the skeletal muscle tissue before and after exercise. The staining for IL-8 in skeletal muscle sections was increased at 2 h postexercise compared with preexercise levels. Further, a proliferation assay was performed to analyze whether CX₃CL1 affected myoblast proliferation. However, CX₃CL1 did not affect myoblast proliferation rate, as measured by BrdU incorporation.

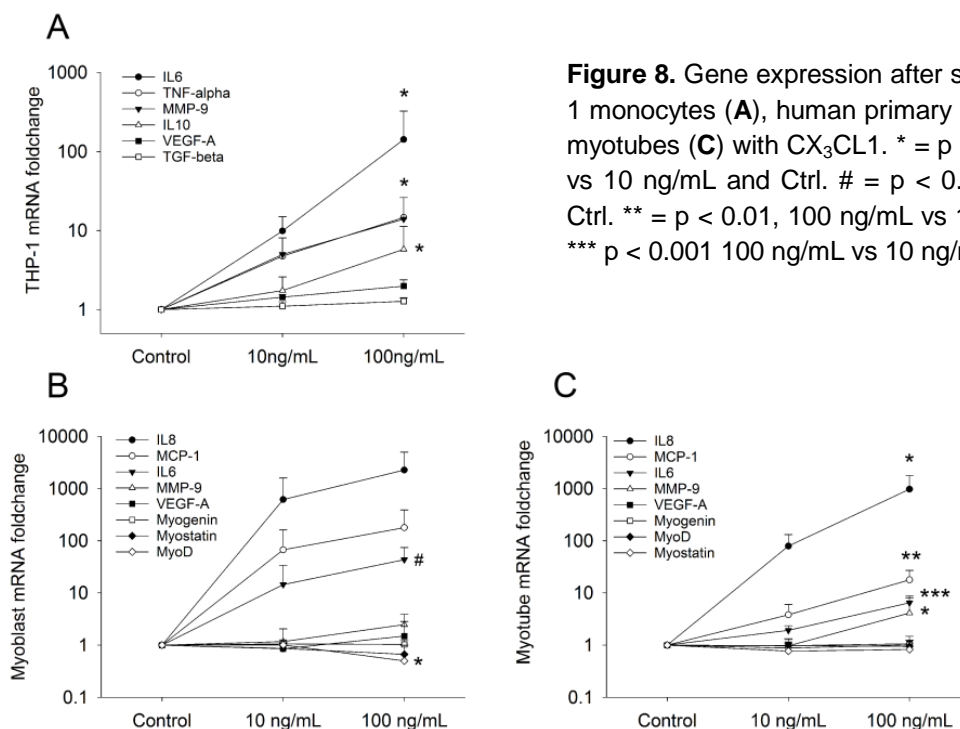


Figure 8. Gene expression after stimulation of THP-1 monocytes (**A**), human primary myoblasts (**B**) and myotubes (**C**) with CX₃CL1. * = $p < 0.05$, 100 ng/mL vs 10 ng/mL and Ctrl. # = $p < 0.05$, 100 ng/mL vs Ctrl. ** = $p < 0.01$, 100 ng/mL vs 10 ng/mL and Ctrl. *** $p < 0.001$ 100 ng/mL vs 10 ng/mL and Ctrl.

5 DISCUSSION

The key findings in this thesis were:

- 1) BM-derived cells are found in human skeletal muscle tissue both as ECs and fused with skeletal muscle fibers
- 2) Monocyte subsets display different temporal patterns in mobilization with exercise
- 3) Monocyte recruiting factors and endothelial adhesion molecules increase in skeletal muscle with exercise
- 4) MMPs increase with exercise with different responses to acute exercise versus a longer period of training.

Data from this thesis also corroborate the idea that cross-talk between different cells types in the muscle tissue, including ECs, muscle fibers and monocytes/macrophages, plays an important role in the adaptation of skeletal muscle to exercise.

5.1 BONE MARROW-DERIVED CELLS IN SKELETAL MUSCLE TISSUE

BM-derived cells have been demonstrated to contribute to skeletal muscle adaptation and regeneration in mice as a physiological response to physical activity or injury (45, 54, 84, 110). Furthermore, monocytes and macrophages have been shown to be crucial for skeletal muscle regeneration. However, the vast majority of research in the field of skeletal muscle adaptation is carried out in non-human systems. To investigate whether BM-derived cells could contribute to the SaC pool, fuse with muscle fibers or form ECs in human skeletal muscle tissue similar to what has been shown in animal models, biopsies were obtained from women transplanted with male BM (Study I). This allowed us to trace BM-derived cells via the Y chromosome. While no contribution to the SaC pool was seen, either in muscle tissue sections nor in myoblasts cultured from the biopsies, Y chromosomes were detected in ECs and in muscle fiber nuclei. This is the first study showing BM-derived contribution to myofibers in humans and, except from a study showing BM-derived ECs in endometrial tissue, the first study showing BM-derived contribution to the endothelium in adult humans (101).

Since Y chromosomes were found in myofiber nuclei but not in the SaC niche, our data support that contribution occurred via fusion to fibers. This is in contrast with one study in mice, where BM-derived cells were found to occupy the SaC niche (84), but corroborates another study where contribution to skeletal muscle occurred via direct fusion (22). We hypothesize that contribution from the BM to myofibers occurs due to a remodeling stimulus, such as exercise or injury. Such a stimulus would recruit circulating cells into the skeletal muscle which would fuse to fibers similar to how myoblasts fuse with myotubes or existing muscle fibers. The phenotype of the cells that had fused with the fibers cannot be elucidated, but since the subjects in our study had about the same level of contribution regardless of whether they had received BM transplantation or peripheral blood stem cells, the origin is likely from the hematopoietic lineage. The extent of contribution in our study was similar to what has been reported in mice, with an average of about 0.7 % of muscle fibers and ECs

containing a Y chromosome. At what time point this contribution occurred after the transplantation cannot be elucidated, but data from mice show that BM-derived cell contribution occurs irrespective of irradiation-induced damage of the skeletal muscle (110). The BM transplanted women in our study performed low-intensity type exercise; their physical activity consisted mainly of walking. Thus, whether a larger contribution would have been seen had these women performed more strenuous type of exercise, similar to what has been reported in mice (84, 110), cannot be excluded. Regardless, due to the low degree of contribution it can be speculated that the importance of BM-derived cells in skeletal muscle remodeling does not lie within the differentiation into/fusion with cells in skeletal muscle tissue, but rather in their capacity to provide a beneficial milieu for adaptation to stress.

5.2 MONOCYTE AND EPC LEVELS IN THE CIRCULATION WITH EXERCISE

In line with our data showing contribution of BM-derived cells to human skeletal muscle, and data from mice showing that this contribution is increased with exercise, we wanted to explore the effect of exercise on the circulating levels of monocytes and EPCs (Study II). These cell subsets are proposed to be important for skeletal muscle remodeling either by contributing to cells in the skeletal muscle tissue or by providing paracrine signaling to the environment. We utilized a flow cytometric protocol designed for enumeration of monocyte subpopulations and EPCs using bead based determination to assess absolute number of cells (64). The distinct monocyte subsets displayed different characteristics in their mobilization to the circulation with exercise. Immediately after exercise, all monocyte subsets were significantly increased with the nonclassical monocytes displaying the highest fold increase (~ 2-fold). At 2 h after exercise, the CD16-positive monocytes were back to pre-exercise levels and only the classical subset was increased. This is an interesting finding from the aspect that earlier studies have shown that CD16-positive monocytes exhibit a different expression of molecules on their surface, which causes their faster adhesion to the endothelium (50). This could thus explain their faster disappearance from the circulation. The increase in monocytes seen directly after exercise supports these cells to originate in the marginal pool from where cells can rapidly be mobilized. This theory has been proposed before, since CD16-positive monocytes were increased in the circulation in humans with just 1 min of intense exercise (134). Since the classical monocytes showed a biphasic response to exercise, one hypothesis could be that different mobilizing stimuli cause their increase in the circulation. In fact, it has been suggested that the second wave of monocytes are mobilized from the BM (50).

Previous studies in humans have used various sets of surface antigens or functional assays to measure the effect of exercise on numbers of putative EPCs. Here we wanted to assess the level of EPCs utilizing a more novel protocol developed for analysis with higher sensitivity and specificity (64). For example, the utilization of a lyse/no-wash protocol instead of using density centrifugation has been shown to increase the number of viable EPCs (18). Further, the protocol employs exclusion of dead cells and the utilization of high-intensity fluorochromes for low-density antigens. Contrary to monocytes, the level of EPCs did not change significantly with exercise. However, there was a higher inter-individual variance in EPC number than monocyte number, and thus there is a risk that the analysis was underpowered. In fact, the post hoc power analysis showed that 14 subjects would have been required to detect a significant effect of exercise on the number of EPCs.

All subpopulations of monocytes have been shown to increase with epinephrine infusion in healthy human subjects, and in mice, norepinephrine increases mobilization of EPCs from the BM (72). In Study II, there was a significant increase in both plasma epinephrine and norepinephrine with exercise (**Table 3**). The plasma level of VEGF-A did not change with exercise (**Table 3**). Without adjustment for hemoconcentration, the levels of G-CSF and SDF-1 were increased in the plasma with exercise. However, after adjustment, neither G-CSF nor SDF-1 plasma levels were changed with exercise (**Table 3**), which indicates that no secretion of either of the factors into plasma occurred with exercise. Still, it can be speculated that if the BM reacts to changed concentration of these factors in the blood, this modest increase caused by hemoconcentration could have an effect. Regardless, even though we cannot rule out the importance of other mobilizing factors, the current finding support earlier reports in that increased plasma concentrations of catecholamines influence monocyte and EPC mobilization (76, 119).

5.3 RECRUITING FACTORS

To assess whether exercise induces factors known to recruit monocytes and EPCs from the circulation, we analyzed the expression of endothelial adhesion molecules and chemoattractants in skeletal muscle. The mRNA levels of E-selectin, ICAM-1 and VCAM-1 were all increased 2 h after exercise. These factors have all been displayed to be regulated at the mRNA level, which means that an increased mRNA level renders an increased protein expression (17, 109, 132). The importance of these molecules for cell recruitment has been assessed in different animal models of inflammation using either adhesion molecule-specific blocking antibodies or mice that are genetically deficient in one or more adhesion molecules (75, 116). To assess whether it was in fact the ECs that expressed these molecules, immunofluorescent staining of muscle sections was performed. Indeed, all three molecules co-localized with the endothelial marker CD31. Previous studies in humans have only addressed increased cells and shed adhesion molecules in the circulation with exercise, while no study has measured the level of adhesion molecules in the skeletal muscle tissue. Thus, our data are novel and supports the idea of recruitment of cells occurring also with endurance-type exercise. Interestingly, it has been proposed that the faster disappearance of CD16-positive monocytes than classical monocytes from the circulation after exercise is due to their higher expression of LFA-1 (the receptor for ICAM-1), which enables firm attachment to ICAM-1 expressing ECs in peripheral tissues (50). Here we add to this hypothesis by showing an increased ICAM-1 expression in skeletal muscle with exercise.

VEGF-A can both increase expression of endothelial adhesion molecules and mediate monocyte and EPC chemotaxis (10, 33, 80). VEGF-A mRNA levels were increased with a single bout of exercise (Study II), and increased VEGF-A expression is a well-characterized phenomenon in the skeletal muscle tissue with exercise (56, 121). When stimulating HUVECs with VEGF-A (Study II), the expression of both E-selectin and VCAM-1 was increased. VEGF-A can thus be one possible mediator behind the upregulation of endothelial adhesion molecules seen with exercise, in addition to serving as a chemotactic agent. Even though it is obvious that other factors from various sources could serve to induce these adhesion molecules, this finding highlights skeletal muscle-generated factors in the upregulation of endothelial adhesion molecules.

When leukocytes roll along the vessel wall, their interaction with endothelial adhesion molecules can be strengthened as a result of leukocyte activation by chemokines. We show that CX₃CL1 was increased in the skeletal muscle after single bout of exercise (Study IV) and further that it was localized to the skeletal muscle endothelium. Interestingly, membrane-bound CX₃CL1 is capable of rapidly inducing firm adhesion of CX3CR1-expressing cells without the presence of selectins or integrins (58, 68). Thus, the presence of CX₃CL1 on skeletal muscle endothelium supports the potential of skeletal muscle to recruit leukocytes expressing CX3CR1, including monocytes, of which CD16-expressing monocytes are shown to express the highest level of CX3CR1 (3). When CD16-expressing monocytes bind to CX₃CL1, they release IL6, CCL2 and MMP-9, factors which in turn can attract other leukocytes and support tissue remodeling (4). Since all these factors are shown to increase in skeletal muscle with a bout of endurance exercise, it is tempting to speculate that CD16 positive monocytes contribute to exercise-induced protein expression in skeletal muscle (27, 113, 124).

In addition to CX₃CL1, other chemokines like SDF-1, IL8 and CCL2 have been shown to be potent activators of monocyte arrest to the endothelium (29, 52, 78, 114). CCL2 was recently displayed to be increased on the mRNA level with one bout of endurance exercise in human skeletal muscle, followed by an increased plasma level of CCL2 (27). We were not able to detect an increase in CCL2 with exercise (Study IV) when all time points were included in the analysis. However, if only the time points pre-exercise and 30 min post-exercise were compared, similar to the study by Catoire et al (27), an increase of CCL2 with exercise relative to controls was observed in Study IV. Thus, our data indirectly support an exercise-induced expression of CCL2, but to establish changes in the expression of this factor, future studies should consider a design that does not use repetitive biopsy sampling.

5.4 CROSS TALK

Several intra- and intercellular signaling events are induced in the skeletal muscle tissue with exercise. An example is the increased expression of VEGF-A in myogenic cells with exercise which in turn affects the ECs leading to increased capillary formation (108). In study II, we demonstrated an increased expression of VCAM-1, ICAM-1 and E-selectin with exercise. These factors can be induced by different mechanisms, but we and others display that VEGF-A is one potential factor stimulating their induction. Thus, this is one example of where one factor induced in muscle fibers with exercise affects the neighboring ECs to express adhesion molecules, which in turn could recruit cells into the muscle tissue.

Further, in study IV, stimulation of HUVECs with microdialysate from exercised muscle tissue increased their expression of CX₃CL1. This indicates that soluble factors in the tissue fluid affect the endothelium to express recruiting factors. When stimulating THP-1 monocytes and primary human myoblasts and myotubes with CX₃CL1, a number of pro-angiogenic and pro-inflammatory factors showed a strong increase in expression. Several of these factors have been shown by us and others to increase in skeletal muscle with exercise. As an example, IL6 in myoblasts, IL8 in myotubes and MMP-9 in monocytes were upregulated with CX₃CL1 stimulation. These factors can in turn recruit leukocytes, act pro-angiogenic on the endothelium and promote ECM remodeling. Here, we focused on the effect of stimulating myogenic cells and monocytes, but several other cell types reside in the

skeletal muscle tissue which can respond to stimuli associated with injury or exercise. For example, it was recently shown that fibroblasts and SaCs cross-talk through direct cell-cell contact, and fibroblasts stimulated muscle regeneration in injured human skeletal muscle (91).

Finally, in study III we demonstrated an increase in MMP-9 with one bout of exercise and MMP-2, MMP-14 and TIMP-1 with training. This indicates that MMPs play a role not only in the phase following a single exercise bout but also in the long-term skeletal muscle remodeling occurring with training. MMPs can regulate the bioavailability of numerous growth factors. In tumor development, MMP-9 has a pro-angiogenic role by increasing the availability of VEGF-A (15). A similar process has been proposed to occur in skeletal muscle, since VEGF-A is shown to increase in the skeletal muscle interstitium already during an endurance exercise bout suggesting a release from preformed VEGF-A and not a transcriptional mechanism (62). In ischemic mouse muscle, MMP-9 was shown to be crucial for increased capillary formation, with BM-derived macrophages being an important source of MMP-9 (73). In the current work, support for such mechanism also in human skeletal muscle is the increased MMP-9 expression in THP monocytes and myotubes stimulated with CX₃CL1, both plausible sources of the rapid increase in MMP-9 with exercise. However, MMP-9 immunoreactivity within skeletal muscle fibers and absence of MMP-9 transcripts within laser-dissected myofibers indicate that skeletal muscle fibers are not the primary source. The protein data in study III show differences in expression and time patterns for MMP-9 and MMP-2, suggesting different regulating mechanisms. A similar pattern is evident in injured mouse muscle, with MMP-9 being induced within 24 h after injury related to the early inflammatory phase, and MMP-2 displaying a slower increase related to fiber regeneration (79). This is also consistent with the differences in the promotor regions of MMP-9 versus MMP-2 and MMP-14 (151), with MMP-9 being responsive to cytokines and MMP-2 and MMP-14 being only modestly affected by growth factors or cytokines (28). No difference was seen in the expression of MMPs between the two conditions (restricted versus nonrestricted blood flow), suggesting that the mechanical stress and not metabolic perturbation was the important regulatory stimulus behind MMP upregulation. This corroborates data from a previous study, where passive leg movement increased the expression of MMP-2 and MMP-9 in the skeletal muscle tissue (62).

6 CONCLUSIONS

The current data supports that, albeit to a small extent, BM-derived cells incorporate into skeletal muscle fibers and endothelium. Further, an endurance bout of exercise results in the mobilization of monocytes and appears to increase the circulating level of EPCs, cells with proposed effects on skeletal muscle remodeling (**Fig 9 A**). Together with the increase of adhesion molecules and chemoattractants in skeletal muscle tissue, this data support that endurance exercise results in recruitment of circulating cells into the skeletal muscle tissue (**Fig 9 B**). Invasion of circulating cells into the tissue is supported by proteases cleaving the ECM, and here we show that MMPs increase in levels and activity in skeletal muscle tissue with exercise (**Fig 9 C**), adding support to the idea of exercise as a remodeling stimulus contributing to recruitment of circulating cells.

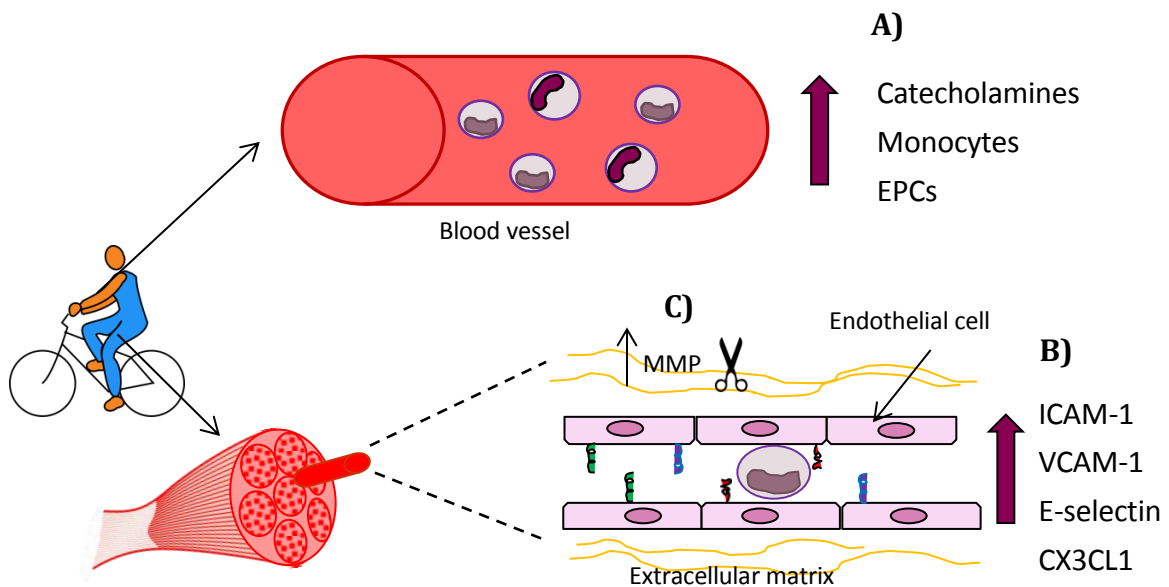


Figure 9. Schematic illustration of the results. With one bout of endurance exercise, the level of monocytes, EPCs and catecholamines were increased in the circulation (**A**). The expression of ICAM-1, VCAM-1, E-selectin and CX3CL1 was increased in skeletal muscle after one bout of endurance exercise and they were all expressed by endothelial cells (**B**). The level of MMPs increased with exercise with different responses to acute exercise versus a longer training period (**C**).

7 FUTURE PERSPECTIVES

Based on the data demonstrated in the current thesis and that of others showing the role of inflammatory processes in skeletal muscle repair and remodeling, it can be speculated that these should not be seen as harmful. In fact, based on data from eccentric exercise in humans, it has been suggested that an increased inflammatory response in skeletal muscle is a part of the repeated bout effect and important for the adaptive response (37). Blocking or reducing inflammatory reactions in the skeletal muscle has been shown to restrict proper remodeling to occur. In mice, depletion of neutrophils or monocytes prevents skeletal muscle regeneration to occur (135, 138). Further, several studies have demonstrated negative effects of non-steroidal anti-inflammatory drugs (NSAIDs) on skeletal muscle regeneration and growth both *in vitro* and *in vivo* (19, 90, 98). This has been partly attributed to a reduced infiltration of macrophages and neutrophils into the skeletal muscle (19). Interestingly, the effect of NSAIDs on skeletal muscle is different between young and old individuals, where aged subjects rather show benefits from NSAID ingestion (142). In a study in rodents, this was interpreted as aging rats having a constant low grade inflammation profiting from medication (122). Whether aging muscle contains and/or recruits a different immune cell pool than young skeletal muscle is, to our knowledge, not known, but it would be interesting to investigate whether such a mechanism could contribute to elderly people showing a different response to NSAID ingestion.

The current thesis did not investigate the number or phenotype of leukocytes in the skeletal muscle tissue. To further study the impact of exercise on either infiltration of cells from the circulation or activation of inflammatory pathways or resident immune cells in the skeletal muscle, caution should be exercised when deciding the time points and number of muscle biopsies. We showed the necessity of including a control group when obtaining biopsies in study IV, where genes were upregulated also in the group of subjects that did not exercise. Further, since other studies in humans have shown that significant increases in immune cells in skeletal muscle occurs 28 h or later after exercise (94, 112), the timing of the post exercise biopsy is important in order to catch any leukocyte accumulation in the skeletal muscle. Regardless of whether circulating cells infiltrate the skeletal muscle tissue, they could have a beneficial role. Nonclassical monocytes have been characterized as cells patrolling the vasculature through binding to ICAM-1 and CX3CL1, which allow them to extravasate into the tissue quickly upon damage (12, 140). It would be interesting to study whether exercise induces an increase in the baseline level of monocytes crawling in the skeletal muscle tissue vasculature.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Skelettmuskulaturen är det organ som utför våra viljestyrda rörelser, och har en stor kapacitet att anpassa sig till förändrade krav. Detta kan illustreras genom den tillväxt av muskulaturen som sker vid styrketräning, eller vid det motsatta förhållandet genom den minskning av muskelmassa som sker vid inaktivitet. Våra muskler är uppbyggda av muskelfibrer, vilka är skelettmuskelns celler. Inuti varje muskelfiber finns proteiner som ansvarar för muskelns förmåga att dra ihop sig. Varje muskelfiber omges av små blodkärl, kapillärer, som försörjer muskelfibrerna med syre och näring och transporterar bort koldioxid och andra ämnen som muskeln bildar vid sin ämnesomsättning. Runt varje fiber finns också ett tunt lager av bindväv, vilket sammanfogar muskelfibrerna med varandra och ger stadga. Vid konditionsträning sker anpassning i muskulaturen genom att det bildas fler kapillärer vilket ökar kapaciteten att transportera syre och näring till muskeln. Dessutom sker en ökad inlagring av glykogen och fett i muskeln, för att öka muskelns energidepåer, samt en ökad förmåga att förbränna dessa energidepåer.

Tidigare har man trott att skelettmuskelns anpassning till förändrade krav endast sker med hjälp av celler som finns inuti muskeln. Dock har det på senare år kommit studier som visar att celler från benmärgen kan bidra genom att ta sig från benmärgen via blodbanan in i muskeln och där hjälpa till i processer såsom bildandet av nya blodkärl, tillväxt av muskelfibrer och skapandet av en miljö i muskeln som främjar denna anpassning. I denna avhandling använde vi oss av olika experimentella modeller för att studera om celler från benmärgen bidrar till skelettmuskelfibrer eller blodkärl i människa. Dessutom har vi undersökt om konditionsarbete i form av ett en timme långt arbetspass på cykel ökar antalet celler i blodbanan som har föreslagits bidra till skelettmuskelns uppbyggnad vid träning. Slutligen har vi studerat olika processer som kan leda till att celler hämtas in i muskeln från blodbanan, även detta efter en timmes cykelarbete.

De försökspersoner som deltagit i dessa studier har varit benmärgstransplanterade kvinnor (Studie I) samt unga, friska frivilliga kvinnor och män (Studie I – IV). Genom att de benmärgstransplanterade kvinnorna i studie I blivit transplanterade med manlig benmärg möjliggjordes analysen av benmärgsceller i deras skelettmuskel. Dessa celler har nämligen Y-kromosomen i sig vilket inte kvinnans egna celler har. För att studera ovan nämnda frågor har vi tagit muskelbiopsier från utsidan av låret samt blodprover. Vi har använt oss av olika metoder för att mäta genuttryck, proteiner samt celler, och genom att göra fina snitt av muskel och färga in dessa snitt har vi kunnat visa på vilka celler i muskeln som har ett protein av intresse på sin yta.

Sammanfattningsvis visar studierna i denna avhandling att celler från benmärgen går att finna i skelettmuskelfibrer och i blodkärlsväggar. Vidare visar vi att konditionsarbete ökar mängden av celler i blodbanan som kan bidra till skelettmuskelns anpassning till träning. Slutligen visar vi att processer som deltar i att transportera celler från blodbanan in i vävnaden aktiveras i skelettmuskeln av ett enstaka konditionsarbetspass.

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You're entirely bonkers.
But I'll tell you a secret,
All the best people are.
— Alice in Wonderland